Serum Turbidity Responses of Anti-Inflammatory Drugs in Endocrine-Deficient Rats

By C. COLOMBO, S. J. PILIERO, and L. GROSSO

The turbidimetric responses of sera to heat from cold stress and endocrine-deficient rats and the effects of phenylbutazone, paramethasone, and adrenocorticotropic hormone (ACTH) have been compared. Sera from cold-stress animals (24 hr.) reveal decreases in the turbidimetric response to heat followed by a return to pre-exposure levels 5 days after cold stress. These responses appear to be operative through the pituitary-adrenocortical axis. Adrenalectomized animals show an inhibition in their serum-turbidity responses to heat which can be prevented by the administration of paramethasone. A further inhibition of protein coagulation by heat occurs in phenylbutazone-treated adrenalectomized rats. This decrease in serum-turbidity readings is similar to that observed in phenylbutazone-treated sham-operated animals. Hypophysectomy or pleuriglandular deficiency causes no protein-stabilization effect to heat which is not attributable to a lack of ACTH. The protein-stabilization effect of phenylbutazone as revealed by the decreases in serum-turbidity responses to heat appears to require an intact pituitary.

RECENTLY, Piliero and Colombo (1, 2) developed an *in vivo* assay for screening drugs for potential anti-inflammatory activity. The method, as well as those procedures described by Mizushima *et al.* (3–5) and Glenn and Kooyers (6), is based on the ability of compounds to inhibit heat denaturation of proteins.

Regarding the underlying mechanism of action of the *in vivo* assay as conducted in normal animals, evidence indicates that the activity of many drugs (nonsteroidal) in the normal animal is through a stabilizing effect on serum-protein coagulation which appears to be operative through a protein-binding phenomenon involving lysyl epsilon-amino groups (2). This appears to be in agreement with the hypothesis of Whitehouse and Skidmore (7) that an important property of nonsteroid anti-inflammatory agents is their ability to uncouple oxidative phosphorylation. In view of the lack of turbidity responses of glucocorticoids in normal animals (2), the present study was undertaken to investigate the role of the pituitary-adrenal axis on turbidity changes in cold-stressed rats and in endocrine-deficient animals treated with adrenocorticotropic hormone (ACTH), paramethasone, and phenylbutazone.

MATERIALS AND METHODS

Four-hundred and thirty-three young adult male rats of the Charles River (CD) strain, weighing approximately 200–225 g. were employed in all experiments. All animals were fed a standard laboratory ration with water *ad libitum* and were kept at temperatures of 70–80° F.

Adrenalectomized animals were maintained on 1% sodium chloride given as drinking water while hypophysectomized animals received supplements of 5% glucose solution. In addition, one group in which adrenalectomy was employed as part of a pleuriglandular procedure (hypophysectomy) received a single maintenance dose of 2.5 mg. of cortisone acetate injected intramuscularly. No accessory adrenal tissue was found to be present in any of these animals at the time of sacrifice.

Serum turbidity (1, 2) was assayed in the following manner: (*a*) To 0.1 ml. of nonhemolyzed serum, 2.9 ml. of 0.066 *M* Sørensen phosphate buffer in saline at pH 5.2 was added and gently agitated. This mixture was allowed to stabilize at room temperature for 15 min. (*b*) The mixture was heated in a Precision constant-temperature shaking water bath set at 69° for 30 min. (*c*) Then, the samples were immediately cooled in an ice water bath and read in a spectrophotometer at a wavelength of 645 μ. Changes in serum turbidity are based on the absorbance readings. Plasma fibrinogen was determined by the method of Goodwin (8). There were four experimental groups.

Intact Animals Following Cold-Stress Exposure (Expt. 1)—Thirty-six animals were subjected to cold stress in a cold room set at 5°. At various intervals, 3, 6, 24, and 120 hr. of continuous exposure, six animals were removed from this stress and immediately sacrificed and exsanguinated for serum-turbidity and plasma-fibrinogen levels in comparison to a group of 12 nonexposed animals. In addition, one group of six rats exposed for 24 hr. was allowed an additional exposure of 3 hr. at room temperature before sacrificing and testing of blood samples.

Hypophysectomized and Control Animals (Expt. 2)—Serum-turbidity studies in three different groups of rats were conducted utilizing 120 hypophysectomized and 135 sham-hypophysectomized

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TABLE I—PLAN OF EXPERIMENT 2

Group	No. of Rats	Agents Used, *Per Os* Unless Indicated	Dose, 3 Hr. Before Sacrifice
Hypox.	20	3% Corn starch	10 ml./kg.
Sham-hypox.	30	3% Corn starch	10 ml./kg.
Hypox. + ACTH	20	ACTH (i.m.)	3 units
Sham-hypox. + ACTH	10	ACTH (i.m.)	3 units
Hypox. + phenylbutazone	20	Phenylbutazone	100 mg./kg.
Sham-hypox. + phenylbutazone	5	Phenylbutazone	100 mg./kg.
Hypox. + ACTH + phenylbutazone	20	ACTH (i.m.); phenylbutazone	3 units; 100 mg./kg.
Sham-hypox. + ACTH + phenylbutazone	10	ACTH (i.m.); phenylbutazone	3 units; 100 mg./kg.
Sham-hypox.	10	3% Corn starch	10 ml /kg.
Sham-hypox. + ACTH	10	ACTH (i.m.)	3 units
Sham-hypox. + phenylbutazone	10	Phenylbutazone	100 mg./kg.
Sham-hypox. + ACTH + phenylbutazone	10	ACTH (i.m.); phenylbutazone	3 units
Hypox.	10	3% Corn starch	10 ml./kg
Sham-hypox.	10	3% Corn starch	10 ml./kg.
Hypox. + paramethasone	10	Paramethasone	1 mg./kg.
Sham-hypox. + paramethasone	10	Paramethasone	1 mg./kg.
Hypox. + phenylbutazone	10	Phenylbutazone	100 mg./kg.
Sham-hypox. + phenylbutazone	10	Phenylbutazone	100 mg./kg.
Hypox. + paramethasone + phenylbutazone	10	Paramethasone; phenylbutazone	1 mg./kg.; 100 mg./kg.
Sham-hypox. + paramethasone + phenylbutazone	10	Paramethasone; phenylbutazone	1 mg./kg.; 100 mg./kg.

animals, operated 6 weeks previously and experimentally treated as indicated in Table I.

Adrenalectomized and Control Animals (Expt. 3)—Serum-turbidity studies in two different groups of rats were conducted utilizing 50 adrenalectomized and 40 sham-operated animals, operated 2 weeks previously and experimentally treated as indicated in Table II.

Hypophysectomized Adrenalectomized and Control Animals (Expt. 4)—Twenty hypophysectomized animals, operated 6 weeks previously were adrenalectomized. After 14 days of adrenalectomy, 100 mg./kg. body weight of phenylbutazone was administered *per os* to 10 of the pleuriglandular deficient rats. Ten other pleuriglandular deficient rats were given 3% corn starch (10 ml./kg.) as controls. Also included were 20 sham-hypophysectomized-adrenalectomized control rats 10 of which were given 3% corn starch (10 ml./kg.) and the remaining 10 animals given phenylbutazone (100 mg./kg. body weight). Serum-turbidity studies were conducted 3 hr. following the administration of the drug.

RESULTS[1] AND DISCUSSION

Changes in protein structure, such as those brought about by denaturation, as a cause of nonspecific inflammation (9, 10) suggests a basic physicochemical action of anti-inflammatory drugs on proteins (2–6). Previous studies employing nonsteroidal and steroidal agents *in vitro* on the turbidimetric response to heat of Cohn fraction IV-4 (3–5) as well as similar *in vivo* studies employing plasma (6) or serum (1, 2) from adjuvant arthritic rats appear to be in accord with this concept. In the authors' serum-turbidity model employing normal animals only the nonsteroidal agents and not the glucocorticoids have been found to be effective in inhibiting protein coagulation by heat as reflected by decreases in absorbance (2).

The present study (Fig. 1) reveals that cold stress (24 hr.) brings about an inhibition of protein coagulation by heat, accompanied by an increase in fibrinogen levels. However, as reflected by the turbidity response a degree of adaptation appears to occur in the 5-day cold-exposed group in that there is at this time a return in values to pre-exposure levels. Apparently, the fibrinogen levels do not reflect to the same degree this adaptation since, although somewhat lower, the high fibrinogen values are still maintained. The data presented appear to be in agreement with the fact that cold stress involves the pituitary-adrenocortical axis (11, 12).

The serum-turbidity levels of the hypophysectomized rats (Fig. 2) reveal significant increases in comparison to the values obtained for sham-operated animals. This seems to imply that the lack of inhibition of protein coagulation by heat in the hypophysectomized animals may be due to the absence of ACTH. However, that ACTH deficiency is not responsible for the lack of protein-coagulation inhibition by heat in the hypophysectomized animal appears to be attested by the fact that the administration of ACTH was without effect in such animals.

Phenylbutazone has been shown to cause a stabilization of the coagulation of serum proteins by heat (1, 2), an effect which is most likely due to its ability to uncouple phosphorylation (7). The effects of phenylbutazone and paramethasone, administered either singly or in combination, in hypophysectomized and sham-operated rats on the levels of serum turbidity are shown in Fig. 3. Of importance is the observation that phenylbutazone does not cause a significant lowering effect in the hypophysectomized animal in comparison

[1] All statements made in this section regarding differences among the means of the different groups of animals compared are based on statistical analyses for probability values derived from the distribution of Fisher's t. Values of $p < 0.05$ have been considered significant.

TABLE II—PLAN OF EXPERIMENT 3

Group	No. of Rats	Agents Used (*Per Os*)	Dose (3 Hr. Before Sacrifice)
Adx.	10	3% Corn starch	10 ml./kg.
Sham-Adx.	10	3% Corn starch	10 ml./kg.
Adx. + phenylbutazone	10	Phenylbutazone	100 mg./kg.
Sham-Adx. + phenylbutazone	10	Phenylbutazone	100 mg./kg.
Adx.	10	3% Corn starch	10 ml./kg.
Sham-Adx.	10	3% Corn starch	10 ml./kg.
Adx. + paramethasone	10	Paramethasone	1 mg./kg.
Adx. + paramethasone + phenylbutazone	10	Paramethasone; phenylbutazone	1 mg./kg.; 100 mg./kg.
Sham-Adx. + phenylbutazone	10	Phenylbutazone	100 mg./kg.

to the evident lowering noted in the sham-operated group. Significant increases in the absorbance readings are noted in both groups of animals receiving paramethasone alone. This seems to indicate that adrenal insufficiency is not the reason for the effect seen in the hypophysectomized group (Fig. 2). On the other hand when phenylbutazone is administered in combination with paramethasone, the rises previously noted with paramethasone alone are prevented in both groups. The administration of phenylbutazone causes an inhibition of serum-protein coagulation by heat in ACTH-treated sham-operated animals but did not affect the lack of stabilization seen in the hypophysectomized group (Fig. 2).

Figure 4 indicates the effects of phenylbutazone in adrenalectomized rats on the levels of serum turbidity. A decrease in absorbance values occurs in the untreated adrenalectomized rats. Further, the administration of phenylbutazone in the adrenalectomized group results in a significant decrease of the absorbance reading, beyond the lowering observed in the untreated adrenalectomized groups, and is of the same magnitude as seen for the treated sham-operated animals.

The effects of the combination of phenylbutazone and paramethasone in adrenalectomized rats on the levels of serum turbidity are shown in Fig. 5. It will be noted that paramethasone in the adrenalectomized rat results in a significant increase in absorbance readings, an effect previously mentioned for both hypophysectomized and sham-operated groups (Fig. 3). The paramethasone effect can possibly be explained on the basis that such substances prevent the uncoupling of phosphorylation (7). On the other hand, the combination of phenylbutazone and paramethasone administered to the adrenalectomized rat results in the prevention of the rise seen with paramethasone alone. In addition, the lowering normally seen in the adrenalectomized, phenylbutazone-treated animal (Fig. 4) does not occur.

The serum-turbidity levels of the hypophysectomized-adrenalectomized rats (Fig. 6) reveal significant increases in the absorbance readings. Figure 6 also indicates that phenylbutazone in

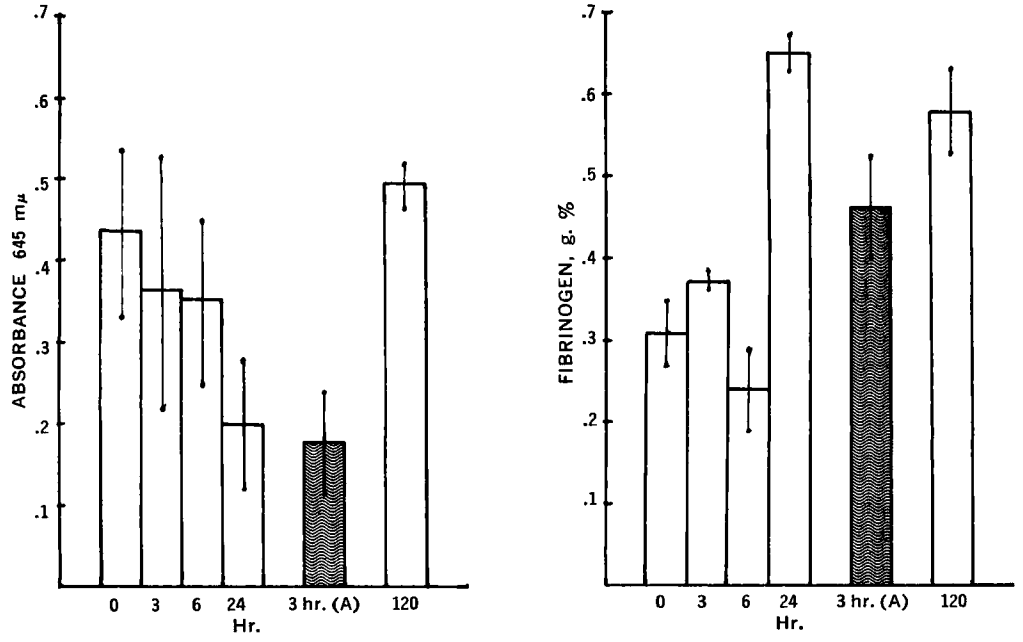

Fig. 1—Effects of cold-stress exposure in rats on the levels of fibrinogen and serum turbidity (absorbance reading, means ± standard errors). Key: A, 3 hr. following 24-hr, cold exposure.

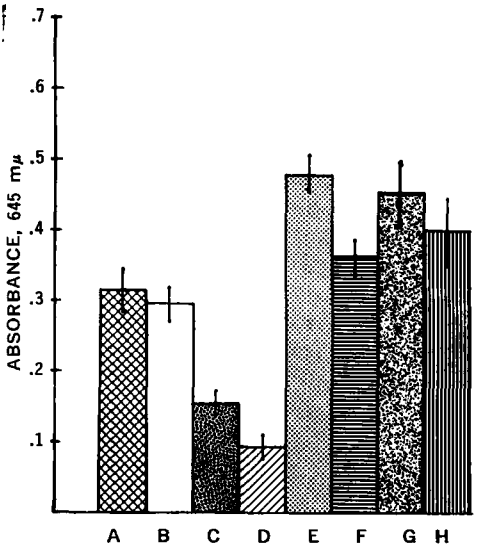

Fig. 2—Effects of phenylbutazone in hypophysectomized and sham-operated rats treated with ACTH (3 units/rat i.m.) on the levels of serum turbidity (absorbance readings, mean ± standard errors). Key: A, sham + ACTH (starch); B, sham control (starch); C, sham + ACTH + phenylbutaz. (100 mg./kg.); D, sham + phenylbutaz. (100 mg./kg.); E, hypox. + ACTH (starch); F, hypox. + ACTH + phenylbutaz. (100 mg./kg.); G, hypox. (starch); H, hypox. + phenylbutaz. (100 mg./kg.).

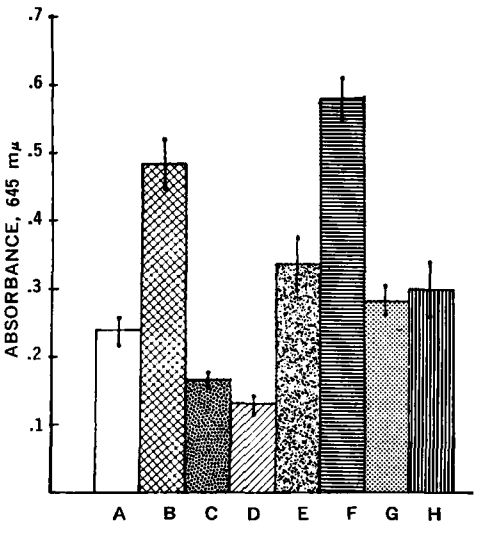

Fig. 3—Effects of phenylbutazone and parametha-sone, administered singly or in combination, in hypophysectomized and sham-operated rats on the levels of serum turbidity (absorbance readings, means ± standard errors). Key: A, sham control (starch); B, sham + parameth. (1 mg./kg.); C, sham + parameth. (1 mg./kg.) + phenylbutaz. (100 mg./kg.); D, sham + phenylbutaz. (100 mg./kg.); E, hypox. (starch); F, hypox. + parameth. (1 mg./kg.); G, hypox. + parameth. (1 mg./kg.) + phenylbutaz. (100 mg./kg.); H, hypox. + phenylbutaz. (100 mg./kg.).

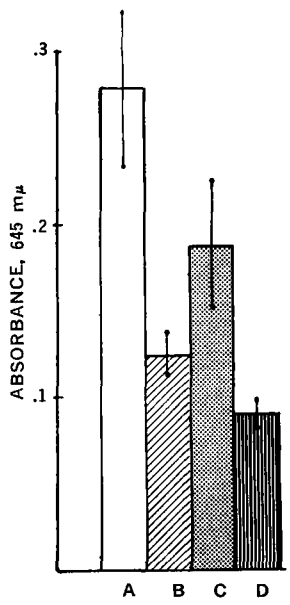

Fig. 4—Effects of phenylbutazone in adrenalectomized rats on the levels of serum turbidity (absorbance readings, means ± standard errors). Key: A, sham control (starch); B, sham + phenylbutaz. (100 mg./kg.); C, adx. (starch); D, adx. + phenylbutaz. (100 mg./kg.).

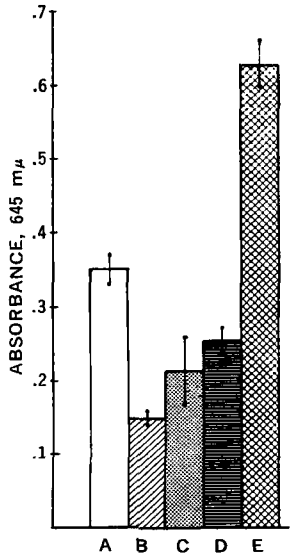

Fig. 5—Effects of phenylbutazone and paramethasone, administered either singly or in combination, in adrenalectomized and sham-operated rats on the levels of serum turbidity (absorbance readings, means ± standard errors). Key: A, sham control (starch); B, sham + phenylbutaz. (100 mg./kg.); C, adx. (starch); D, adx. + parameth. (1 mg./kg.) + phenylbutaz. (100 mg./kg.); E, adx. + parameth. (1 mg./kg.).

such pleuriglandular deficient animals is ineffective in changing the response seen in the phenylbutazone-treated sham-operated group. As previously cited for the hypophysectomized group (Fig. 3), the above results infer that an intact pituitary is

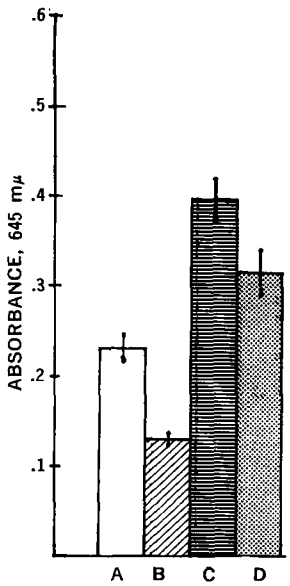

Fig. 6—Effects of phenylbutazone in hypophysectomized-adrenalectomized and sham-operated rats on the levels of serum turbidity (absorbance readings, means ± standard errors). Key: A, sham control (starch); B, sham + phenylbutaz. (100 mg./kg.); C, hypox. + adx. (starch); D, hypox. + adx. + phenylbutaz. (100 mg./kg.).

necessary for the stabilizing action of phenylbutazone.

The results of the present study do not preclude the possibility that endocrines other than the pituitary-adrenal system and/or enzyme systems are involved in the serum-turbidity responses of drugs to heat.

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Keyphrases

Anti-inflammatory activity—screening method
Serum turbidimetric response—heat application
Turbidimetric response, heat—anti-inflammatory agents
Adrenal gland effect—turbidimetric response
Pituitary gland effect—turbidimetric response
Absorbance—analysis, turbidity

Structure-Activity Relationships Among Some Selected Substituted Cyanoacetamides

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Selected *N*-alkyl- and *N,N*-dialkyl-2,2-dialkylcyanoacetamides, including 2-cyanoacyl-, piperidines, pyrrolidines, and morpholines, were synthesized in order to study structure-activity relationships. These were administered to rats and their anticonvulsant activity evaluated by means of the electroshock test. The median effective dose was determined for seven compounds exhibiting protective action, while seven were inactive or displayed convulsant properties. An estimated LD_{50} was determined for the three most effective and least toxic substances. Preliminary pharmacological data have been tabulated and correlations of bioaction with structure are presented.

THE DEMONSTRATION of anticonvulsant activity within a series of 2,2-dialkylcyanoacetamides (1, 2) and in particular, the significant activity of 2-ethyl-2-propylcyanoacetamide and of 2,2-diethylcyanoacetamide against both supramaximal electroshock and subcutaneous pentylenetetrazol led to the consideration of these amides as suitable parent compounds upon which to effect molecular modifications that might serve

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